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Epstein–Barr Viral DNA in Tissues of Hodgkin's Disease

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Tissue specimens from 21 cases of Hodgkin's disease were examined for the presence of Epstein–Barr virus (EBV) and cytomegalovirus DNA by molecular hybridization techniques. EBV DNA was detected in 4 cases, including 2 of 8 cases which had been previously shown to contain clonal immunoglobulin gene rearrangements. Two of the cases containing EBV DNA were of the nodular sclerosing type and had received prior therapy; the other 2 were classified as mixed cellularity Hodgkin's disease and had not received therapy before the biopsy tissue was obtained. Analyses of the terminal portions of EBV genomes indicated a monoclonal or oligoclonal proliferation of EBV-infected cells in the tissues studied. In contrast, none of the 21 cases had detectable cytomegalovirus DNA sequences. The identification of EBV DNA may reflect the proliferation of lymphoblastoid cells due to the reduced immune competence frequently noted in Hodgkin's disease or may indicate the presence of EBV genomes within Reed–Sternberg cells. (Am J Pathol 1987, 129:86–91)

RECENTLY we reported the detection of clonal immunoglobulin gene rearrangements within tissues from certain cases of Hodgkin's disease.[1] All of these cases were of the nodular sclerosing type, and rearrangements were found far more frequently in those tissues that contained large numbers of Reed–Sternberg cells (RS cells). The correlation of detectable immunoglobulin gene rearrangements with large numbers of RS cells suggested that the rearrangements that we found were present in the malignant RS cells. However, our data did not exclude the origin of the rearrangements within the B cells that were present in low numbers within the tissues analyzed. Possibly there was hidden among these B cells a small clonal population that contained uniform gene rearrangements and escaped detection in immunophenotypic studies.

A number of explanations could account for clonal B-cell populations in Hodgkin's disease. Among these is that infection by Epstein–Barr virus (EBV) had induced a clonal proliferation of lymphoblastoid cells in the setting of impaired immunity often associated with Hodgkin's disease. This situation is similar to that observed among immunosuppressed transplant patients in whom clonal lymphoproliferative disorders are apparently caused by EBV.[2–5]

To investigate this possibility we analyzed the DNA of tissue from 21 cases of Hodgkin's disease, including those specimens that earlier showed clonal immunoglobulin gene rearrangements, for the presence of EBV genomes. Our results indicate that EBV DNA can be found in tissues from some cases of Hodgkin's disease. However, EBV DNA was detected both in those cases that showed clonal gene rearrangements and in those that lacked rearrangements; and included among the cases containing EBV DNA were examples of both the nodular sclerosing and mixed cellularity types.

Materials and Methods

Twenty-one cases of Hodgkin's disease were studied; 8 cases were part of our previous study of immunoglobulin and T-cell receptor gene rearrangements in Hodgkin's disease tissues containing abundant RS cells.[1] All cases were morphologically diagnostic of

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Hodgkin's disease. In addition, the 8 cases from the previous study were studied by frozen-section immunoperoxidase techniques and were found to have immunophenotypes compatible with Hodgkin's disease. In no case were the histologic or immunophenotypic findings thought to indicate a malignant lymphocytic component suggesting a composite Hodgkin's/non-Hodgkin's lymphoma. Among the 21 cases studied, 13 cases were of the nodular sclerosing type, 4 cases were of the mixed cellularity type, and 4 cases were of the lymphocyte-predominance type of Hodgkin's disease. In 9 cases the initial biopsies were studied, in 11 cases recurrences were studied, and in 1 case both initial and recurrent specimens were studied. Lymphoid tissue from sites which histologically lacked Hodgkin's disease were also analyzed in 2 cases.

DNA was extracted from frozen tissues according to methods previously described.[6,7] For the slot blot hybridization studies, 5 μg of DNA purified from each tissue specimen was applied to nylon membranes by means of a slot blot application device[8]; the DNA immobilized on the membrane was then hybridized with an EBV Bam HI-W[9] or Eco RI-B[10] fragment of EBV DNA that had been radiolabeled by the random hexamer priming technique.[11] Slot blots for detecting cytomegalovirus genomes were hybridized with the 12-kilobase viral DNA fragment of the cosmid pCM1039.[12] The Southern blot hybridization studies were performed as described elsewhere.[6] Ten micrograms of purified DNA was digested by Bam HI restriction enzyme, and the resulting fragments were size-fractionated by electrophoresis and transferred onto activated nylon membranes.[13] The membranes were hybridized with probes by methods described elsewhere.[6] In addition to Bam HI-W and Eco RI-B probes, Southern blots were also hybridized with a 5.2-kilobase Bam HI-Eco RI restriction fragment containing the 500 basepair tandem terminal repeated sequences of the EBV genome.[14]

Results

The results of the slot blot hybridizations are shown in Figure 1. DNA was initially screened with the Bam HI-W probe. This probe contains DNA of a region of the EBV genome that is variably iterated in the genome of different viral isolates.[15] The Bam HI-W probe was selected because it should detect EBV DNA more sensitively than a probe specific for single copy sequences. Four of the 21 cases (Cases 2, 5, 13, and 19; 19% of the total) showed hybridization for EBV genomes. Two of 10 initial diagnostic specimens and 2 of 12 recurrent specimens (including 2 of 8 cases from our previous gene rearrangement study) were positive. The histologic subtype in the positive cases was nodular sclerosing in 2 cases and mixed cellularity in 2 cases. In 2 of the 4 cases (Cases 13 and 19), uninvolved tissues were available for study and showed no evidence of EBV genomes.

To roughly quantitate the number of EBV genomes present in the positive cases, we used an Eco RI-B probe. This probe detects a single copy DNA sequence in EBV DNA.[11] Using this probe 3 of the 4 positive cases showed between 1 and about 3 EBV genome equivalents per cell, but EBV DNA could not be detected in the fourth case (Case 19), which had shown the lowest level of hybridization with the Bam HI-W probe. All of the DNAs in this study were also screened for cytomegalovirus genomes and failed to show any hybridization above background levels.

The positive cases as determined by slot blot hybridizations were then confirmed by Southern blot hybridization to ensure that our slot blot analyses were detecting actual EBV genomes and not some cross-hybridizing DNA. As seen in Figure 2, each of the positive cases demonstrated a band corresponding to a roughly 3.0-kilobase DNA fragment by hybridization of Bam HI digests to the Bam HI-W probe, with a relative intensity of bands similar to that seen in the slot blot analyses. The position of this band matched

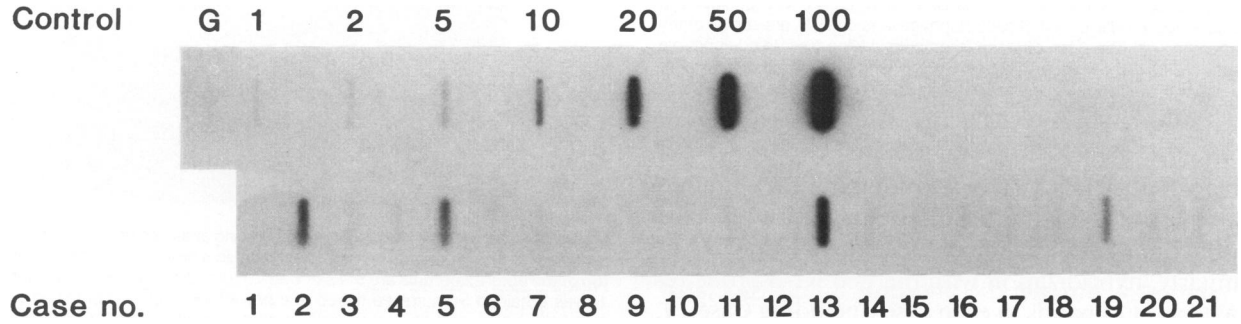

Figure 1—Slot blot hybridization analyses of EBV DNA in tissues from 21 cases of Hodgkin's disease. The DNA was hybridized with the Bam HI-W probe. Controls consisted of germline DNA (G) and varying amounts of Bam HI-W DNA corresponding to between one and 100 copies, as indicated. Because DNA homologous to the Bam HI-W probe is iterated within the EBV genome to different extents in different viral isolates, the results in this figure cannot be used to accurately quantitate the number of EBV genomes in the tissues analyzed.

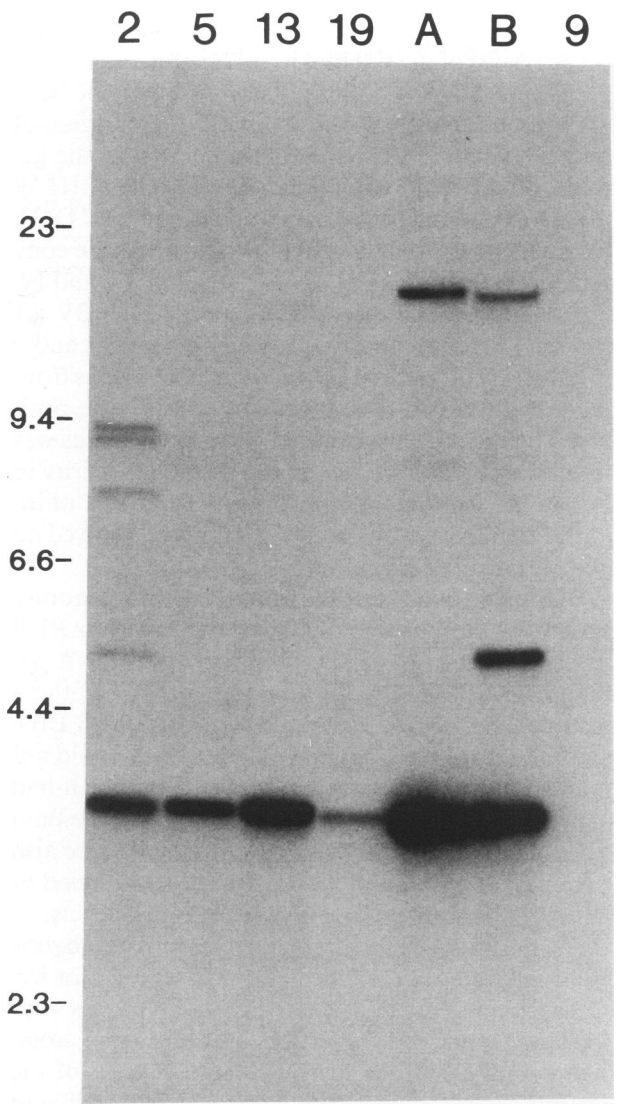

Figure 2—Southern blot autoradiogram showing EBV DNA in tissues from patients with Hodgkin's disease. Numbers at the top of the autoradiogram correspond to case numbers in Figure 1. **A** and **B** represent two positive controls consisting of tissue or cells known to contain high numbers of EBV genomes. **A** was obtained from the spleen of a patient with severe combined immune deficiency who died of an EBV-associated lymphoproliferative disorder.[17] **B** was obtained from a cell line (AW-Ramos) of Burkitt's lymphoma infected with EBV.[36] The DNA in each lane was digested with Bam HI restriction enzyme and hybridized with the Bam HI-W probe. The positions of fragments in a Hind III digest of bacteriophage lambda DNA are shown at the left together with the sizes of these fragments in kilobases. In addition to a roughly 3.0-kb band in all but Case 9, several of the specimens show other bands, which are probably due to DNA polymorphisms in or around the Bam HI-W region of EBV DNA.

that in control DNA known to contain EBV genomes and is consistent with the approximate size reported for the Bam HI-W fragment of isolated EBV DNA.[15] Similarly, hybridization with the Eco RI-B probe revealed a set of bands in each case (including Case 19, which had been negative in slot blot studies with the Eco RI-B probe), indicating the presence of EBV genomes.

Southern blots of Bam HI DNA digests were hybridized with the 5.2-kb probe for the terminal sequences of the EBV genome for assessment of the clonality of the cells containing EBV genomes. This probe detects a polymorphic restriction fragment corresponding to the fused termini of the circular EBV genome found in latently infected cells.[14] Clonal populations of cells derived from a single parent cell infected at low multiplicity of virus will contain a fused terminal EBV DNA fragment of uniform size detectable as a single band in a Southern blot hybridization analysis. The position of this band varies among different EBV-infected clonal populations, depending on the number of tandemly repeated 500-basepair segments of DNA included when the ends of the linear genome recombine after entry of the linear viral genome into the cell. Figure 3 indicates that 3 of the 4 cases of Hodgkin's disease analyzed by us (Cases 2, 5, and 19) contain monoclonal populations of EBV-infected cells, as indicated by a single band in a Southern blot autoradiogram. Case 13 shows two relatively dark bands and a third less intense band at a slightly higher position in the autoradiogram. This may indicate that as many as three clonal populations are

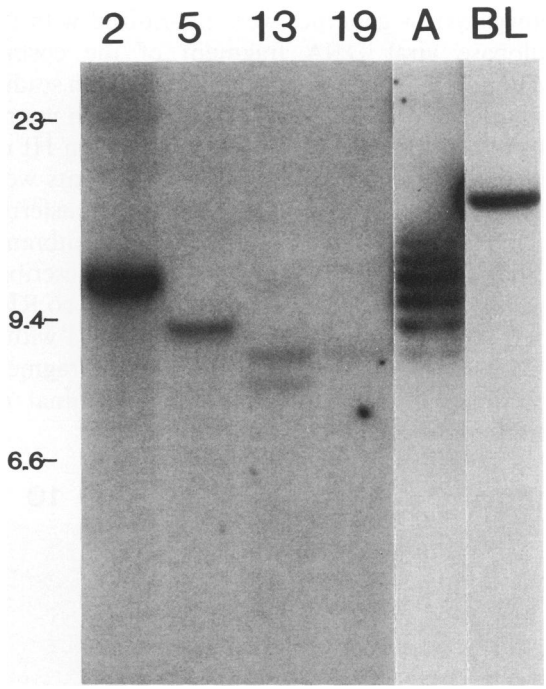

Figure 3—Southern blot autoradiogram showing analyses of fused terminal portion of the EBV genome in tissues of Hodgkin's disease. Numbers at the top of the autoradiograms are the same as in Figures 1 and 2. **A**, as in Figure 1, was obtained from spleen tissue of a patient with severe combined immunodeficiency, and shows a band pattern consistent with a polyclonal proliferation of EBV-infected cells.[17] **BL** was obtained from a tumor in a patient with Burkitt's lymphoma. The DNA in each lane was digested with Bam HI restriction enzyme and hybridized with a 5.2 kilobase Bam HI-Eco RI DNA fragment specific for the termini of the EBV genome.

present, but it may also mean that a single cell was originally infected at high multiplicity such that each cell within one clone contains as many as three different forms of the circular EBV genome.

The case histories of the positive cases are summarized below.

Case 2

This 10-year-old Hispanic boy was diagnosed as having nodular sclerosing Hodgkin's disease in a left axillary lymph node biopsy 5 years previously. At that time, he was inadequately staged and was treated with an 8-day course of radiation therapy. At the time of recurrence, he presented with nephrotic syndrome as well as left axillary lymphadenopathy. Biopsy of a left axillary lymph node (the specimen analyzed in the current study) revealed recurrent Hodgkin's disease. The patient was staged as IIIA because of the presence of abdominal masses and was treated with combined modality therapy consisting of MOP(P) with ABVD as well as total lymphoid irradiation. He shows no evidence of disease 13 months after completion of therapy.

Case 5

This 29-year-old white man presented with a pruritic rash and fevers and was given a diagnosis of nodular sclerosing Hodgkin's disease as a result of a left supraclavicular lymph node biopsy 3 years previously. He was staged as IIISB on the basis of splenic involvement and was treated with mantle field radiation therapy and PAVe chemotherapy, with complete response. Two years after completion of therapy, he developed left inguinal lymphadenopathy, which a biopsy showed to be recurrent nodular sclerosing Hodgkin's disease (the specimen analyzed in the current study). He was then treated with MOPP and ABVD chemotherapy and shows no evidence of disease 7 months after completion of therapy.

Case 13

This 22-year-old white man noted a left axillary mass which on biopsy revealed mixed cellularity Hodgkin's disease. He was staged as IIISA-unfavorable on the basis of bulky splenic involvement; a splenic hilar lymph node was also found to be involved. Both an involved splenic nodule and uninvolved splenic tissue were analyzed in the current study. The patient was treated with mantle radiotherapy and PAVe chemotherapy and shows no evidence of diseases 3 years after completion of therapy.

Case 19

This 54-year-old Japanese-American woman with a longstanding history of rheumatoid arthritis presented with diffuse lymphadenopathy; biopsy of a right cervical lymph node revealed mixed cellularity Hodgkin's disease. Staging laparotomy demonstrated massive splenic involvement, as well as involvement of several splenic hilar, para-aortic, and celiac lymph nodes. Both involved and uninvolved paraaortic lymph nodes were analyzed in this case. The patient was staged as IIISA and treated with MOPP/ABVD chemotherapy. The patient shows no evidence of disease 11 months since completion of therapy.

In none of these 4 cases was there a history of preceding or concomitant EBV-related infection nor are antibody studies available in any of the cases. Tissues from Cases 2 and 5 had previously shown clonal immunoglobulin gene rearrangements,[1] while tissues from Cases 13 and 19 had not.

Discussion

Our findings suggest a meaningful association between Hodgkin's disease and EBV. Normal tissues, with the exception of salivary glands, have generally been found to be negative for EBV DNA by hybridization methods.[16,17] Furthermore, DNA from 30 non-Hodgkin's lymphomas not arising in immunocompromised hosts have been studied in our laboratory and showed no detectable EBV genomes (Cleary and Sklar, unpublished observations). Therefore, it is likely that the EBV genomes detected in the tissues we studied are related to the presence of Hodgkin's disease and are not simply a fortuitous finding. Previous failure to identify EBV genomes in Hodgkin's disease may be attributable to use of less sensitive methods involving hybridization of cRNA probes to tissue DNA trapped on nitrocellulose filters.[18,19]

Two explanations seem plausible for the detection of EBV DNA in tissues of Hodgkin's disease. One explanation is that EBV may be present for the reason outlined in the Introduction, namely, because of the proliferation of B lymphoblastoid cells carrying EBV genomes. Proliferation of B lymphoblastoid cells might be tolerated because of defective immunologic surveillance in patients with Hodgkin's disease. However, several observations argue against this hypothesis. For instance, the lack of detectable cytomegalovirus genomes in the cases suggests that the EBV genomes that were found are not merely a manifestation of depressed immune function. More importantly, our failure to identify EBV genomes in uninvolved tissues from 2 patients in whom genomes were detected in involved sites would also support the no-

tion that a generalized defect in immune surveillance is not responsible for the presence of the viral genomes. To the contrary, these data imply that the relationship of Hodgkin's disease and EBV is an anatomically local one, especially as evidenced by Case 13, in which a splenic nodule involved with Hodgkin's disease showed EBV genomes, but tissue of nearby uninvolved splenic parenchyma did not.

The results on the clonality of the EBV-infected cells also seem to be at odds with an explanation for our findings based on depressed immunity in Hodgkin's disease. If depressed immunity underlies the presence of EBV DNA in these tissues, then lymphoblastoid cells would be expected to be the carriers of the viral DNA. Studies of the termini of the EBV genome in infected tissues indicated monoclonal or oligoclonal proliferations of infected cells. But these cells are probably not lymphoblastoid cells, because clonal proliferations of such cells should be detected as uniform immunoglobulin gene rearrangements, and only half of the cases that contained EBV genomes in our study showed clonal rearrangements of these genes. These results contrast with the findings in immunosuppressed transplant patients with EBV-associated lymphoproliferations.[2-5,20] Individual lesions in such patients usually show monoclonal or oligoclonal patterns of immmunoglobulin gene rearrangements. In this context, however, it should be noted that the hybridization probe used to analyze the termini of the EBV genome may be more sensitive than the probes for immunoglobulin genes, because this EBV probe detects a repeated sequence within the viral DNA, and each infected cell probably carries multiple copies of the EBV genome. Therefore, although clonal immunoglobulin gene rearrangements were carefully sought in these cases of Hodgkin's disease by using different combinations of probes and restriction enzymes together with long exposure times for autoradiograms, it is still conceivable that a minor clonal population of lymphoblastoid cells was missed because it composed too small a fraction of the total cells in the tissues to be detected by the Southern blot procedure.

A second explanation for the presence of EBV genomes in tissues of Hodgkin's disease is that the virus is located within RS cells, the neoplastic component of Hodgkin's disease tissue, and may even be causally related to this neoplasm. A viral etiology for Hodgkin's disease has in fact previously been suggested because of results from a number of different studies. For example, epidemiologic studies have shown a peak of incidence in young adulthood among economically advantaged populations, while in economically disadvantaged populations no such peak is seen.[21] Also, children in whom Hodgkin's disease develops tend to be from lower social classes.[22] These findings have led to the hypothesis that Hodgkin's disease may arise as a rare consequence of infection by a common virus and that the risk is markedly increased when infection occurs after childhood, similar to what is observed with infections by polio virus.[23] Supporting this hypothesis, the risk of development of Hodgkin's disease is associated with small family size and single family housing in childhood, factors that may delay initial exposure to the etiologic virus.[24]

Data have also accumulated more specifically linking EBV to the cause of Hodgkin's disease. Patients with infectious mononucleosis due to EBV have a two to four times increased risk of Hodgkin's disease.[24,25] In addition, patients with Hodgkin's disease often have higher titers of antibodies against the viral capsid antigen of EBV and higher levels of antibodies against the early antigen than controls.[26,27] Recently, EBV nuclear antigen could be demonstrated in the nuclei of RS-like cells in a lymph node that morphologically resembled Hodgkin's disease from a patient with a chronic EBV infection.[28]

Of course, any argument that EBV is directly involved in the cause of Hodgkin's disease must contend with the fact that EBV DNA is detectable only in a minority of Hodgkin's disease cases. On the other hand, that EBV genomes may be present within RS cells in certain cases of Hodgkin's disease receives some indirect support from data in our study. Analyses of the termini of the EBV genome indicate clonal proliferations of EBV-infected cells within the tumor tissues. The only cells which are invariably clonal in all of the cases containing EBV are the RS cells, because these cells constitute the neoplastic cells in tissues of Hodgkin's disease. The simplest interpretation of these data is that RS cells contain the EBV genomes when EBV is present in Hodgkin's disease tissues. Naturally, this reasoning is subject to the same considerations concerning the superior sensitivity of the probe for the termini of the EBV genome, as discussed above.

If EBV genomes are actually present in RS cells, it is not obvious how they would get there. EBV normally enters B lymphocytes by first binding to the C3d receptor.[29-31] This receptor has also recently been found on the surfaces of certain replicating epithelial cells both *in vitro* and *in vivo*,[32,33] possibly accounting for the presence of EBV within tissues of nasopharyngeal, tonsilar, and thymic carcinomas. However, RS cells lack C3d receptors, as determined by the absence of reactivity with anti-B2 monoclonal antibody.[34] Perhaps an alternative mechanism of entry exists for RS cells. For example, EBV can induce fusion of infected

B lymphoblastoid cells with noninfected receptor-negative cells *in vitro*.[35] Possibly a similar process could occur *in vivo* to introduce EBV DNA into RS cells and may even contribute to the large multilobated nuclear morphology of these cells.

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